METHOD AND MEANS FOR PRODUCING THERAPEUTICALLY EFFECTIVE BLOOD COMPOSITIONS

FIELD OF THE INVENTION

[0001] The invention substantially relates to a process for producing induced blood compositions from blood, wherein the blood cells express, and possibly secrete, transiently or stably, one or more therapeutically or diagnostically important proteins and/or effector molecules.

BACKGROUND OF THE INVENTION

[0002] The production of eukaryotic, recombinant proteins is expensive and costly. The correct processing of such proteins likewise entails problems. The strength of the therapeutic action of the proteins depends strongly on the processing of these proteins. Conventional recombinant proteins show lack of effectiveness due to erroneous processing, for example, failure of glycosylation. The correct processing of recombinant proteins increases the therapeutic effectiveness many times.

[0003] The production of recombinant proteins without impurities is very costly. However, the degree of purity plays a large part in their action and in avoiding side effects. Recombinant proteins produced in the conventional manner can lead to incompatibility reactions, which in particular can be ascribed to the impurities themselves contained in the protein preparations and also to possible reactions of the organism, particularly immune reactions, to the proteins and/or the impurities. The preparation of recombinant proteins recovered from, in particular autologous, cells of the animal or human body, and which are furthermore easily obtainable, is therefore sought after.

[0004] Heretofore, the transfection of different specific fractions from blood cells is known. Van Tendeloo et al. (Gene Therapy (2000) 7:1431-1437) describe systems

for gene transfer in primary human blood cells by means of electroporation, as blood cells activated human T-lymphocytes and/or adult bone marrow cells of type $CD34^+$ being transfected by electroporation, respectively in their cultivated form. Respectively before transfection, the T-lymphocytes are activated by the use of PHA or by CD3 cross linking, and by interleukin-2 before transfection. Likewise, Harrison et al. (BioTechniques (1995) 19: 816-823) describe gene transfer by means of cationic lipids in cell lines or in primary human cells, particularly mononuclear cells of peripheral blood and/or $CD34^+$ enriched hematopoietic cells.

[0005] Alternative methods for transfection of eukaryotic cells, for example by primary mechanical stressing, are described. Costanzo and Fox (Genetics (1988) 120:667-670) describe the transformation of yeast cells with plasmid DNA and small glass beads in YPD medium. The glass beads are used in order to mechanically "wear away" the cells, facilitating the penetration of the plasmid DNA into the yeast cells.

BRIEF SUMMARY OF THE INVENTION

[0006] Starting from the prior art, the technical problem forming the basis of the present invention consists of the provision of methods and means for performing them, making it possible to obtain eukaryotic, therapeutic and/or diagnostic important proteins, particularly autologous proteins, from blood cells, particularly autologous blood cells.

[0007] The technical problem is substantially solved by a transient genetic transformation of blood cells, the cultivation of these cells in serum preferably following directly, and the application of the produced proteins in serum, preferably following, particularly without purification. According to the invention, the problem is solved by a method of production of an induced blood composition from blood, in particular whole blood, that is, blood which has not been purified and/or fractionated, transiently, that is, limited to a given time period, or stably, that is, permanently, transformed with at least one nucleic acid molecule, particularly DNA or RNA. Preferred according to the invention, the at least one nucleic acid molecule preferably codes for at least one gene product, preferably a protein, and particularly preferably a therapeutically and or

diagnostically important protein and/or at least one effector molecule, for example, an effector protein, or preferably at least one nucleic acid molecule, particularly RNA, which modulates, preferably increases, the serum concentration of therapeutically or diagnostically relevant proteins. In a further step of the method according to the invention, a thus induced blood composition is obtained which comprises at least one transformed blood cell which transiently or stably expresses at least one gene product, in particular a therapeutically and/or diagnostically important protein and/or effector molecule, expressed transiently or stably and preferably possibly releases, i.e., secretes, this and, particularly in a further step, from the induced blood composition at least one gene product, preferably the therapeutically and/or diagnostically important protein and/or effector molecule and/or at least one therapeutically and/or diagnostically important protein regulated by an effector molecule is obtained.

[0008] It is particularly advantageous that the use of blood as raw material on the one hand and as a production system for the at least one gene product on the other hand, preferably together with an application of the at least one preferred produced protein particularly in serum is especially simple and cost-favorable. It is also found that the eukaryotic blood cells process the proteins correctly. Furthermore, particularly advantageously, no impurities or other immunogenic components arise in the preferably autologous or heterologous production system according to the invention, which could lead to incompatibility reactions in a receiver of the gene product. In the use of an effector molecule obtained according to the invention, that is, a gene product which in particular modulates, particularly increases, the serum concentration of therapeutically and/or diagnostically important proteins, the blood composition particularly advantageously contains only proteins which are autologous, i.e., derive only from the donor, and are therapeutically and/or diagnostically important. An immunological reaction on application, preferably re-application, of the thus produced blood composition is therefore practically to be excluded.

[0009] If at least one nucleic acid molecule coding for at least one effector molecule, in particular effector protein, is used for the transformation according to the invention, which excites the production of numerous, preferably every, proteins, a

particularly advantageous result is that the production and/or liberation of plural simultaneous, preferably autologous, proteins in the blood cells is excited. In particular, the proteins are then produced and/or liberated in a natural quantitative ratio to one another. Examples of such effector molecules are transcription factors, proteins which are part of a signal transduction chain, extracellular signal molecules such as cytokines, or anti-sense RNAs. Since many diseases have multi-factor causes, the production, particularly simultaneously, of plural therapeutically effective proteins in an individual, physiological ratio of the proteins to one another is particularly advantageous for the use of these proteins as effective materials, particularly in a pharmaceutical composition for treatment of the animal or human body.

[0010] The invention is described in detail using the following examples and Figures.

BRIEF DESCRIPTION OF THE DRAWINGS

[0011] Figure 1is a diagram of the IL-1ß content in the blood cells according to the pyrogenicity test of the plasmid *pcDNA-IL-1Ra*.

[0012] Figure 2 is a graph showing the dependence of DNA binding to glass beads on the pH value of the buffer.

[0013] Figure 3 is a graph showing the dependence of DNA binding to glass beads on the incubation time in DNA-containing buffer.

[0014] Figure 4 shows an agarose gel (2%) after PCR on a dilution series of *pcDNA-IL-1Ra* in TE and on bead wash water. Ethidium bromide staining. The arrows show the comparable dilution steps.

[0015] Figure 5 shows an agarose gel (2%) after PCR on a dilution series of *pcDNA-IL-1Ra* in human serum. Ethidium bromide staining; the control contains no "template" DNA ("water control").

[0016] Figure 6 shows an agarose gel (2%) after a "nested" PCR on a dilution series of *pcDNA-IL-1Ra* in human serum. Ethidium bromide staining; the control contains no "template" DNA ("water control").

[0017] Figure 7 shows an agarose gel (2%) after PCR on a dilution series of *pcDNA-IL-1Ra* in human serum. Sample material of the syringe system according to the invention with "bead transfection," and of the ORTHOKIN ® System. Ethidium bromide staining.

[0018] Figure 8 is a diagram showing the *IL-1Ra* content after "bead transfection" in the blood cell culture.

[0019] Figure 9 shows a diagram of the IL-1Ra content in the serum supernatant after 24 hours' incubation of whole blood. Comparison of the syringe system according to the invention with "bead transfection" with the ORTHOKIN® system, a perfusion syringe without beads and with the control sample.

[0020] Figure 10 shows a diagram of the specific activity of ß-galactosidase after electroporation of PBMCs with *pVaxLacZ*.

[0021] Figure 11 shows a schematic diagram of a syringe (1) used according to the invention, of glass or plastic, with a plunger (3), an optional screw-on closure (5), a closure shoulder (13), and arranged thereon and closing this a removable cap (7) with septum. The plunger (3) preferably has a fracture place (15). Shown is also a coated granulate (9) according to the invention, in the lumen of the syringe.

DETAILED DESCRIPTION OF THE INVENTION

[0022] In a particular embodiment of the method according to the invention, the blood composition induced according to the invention has at least one therapeutically and/or diagnostically important protein in higher concentration than in a non-transformed blood composition. Preferred according to the invention are therapeutically and/or diagnostically important proteins such as cytokines such as the natural or modified interleukin-1 receptor antagonist, IL-1Ra (IRAP).

[0023] Preferred according to the invention, expressed in at least one blood cell, is the blood composition induced according to the invention of at least one effector molecule, particularly effector protein, which in non-transformed blood cells is not expressed at all, or is expressed in an amount which lies below that level which is

produced by the at least one blood cell expressing the effector molecule, particularly effector protein, of the blood composition produced according to the invention.

[0024] In a further preferred embodiment of the method according to the invention, the blood for producing the induced blood composition is taken from an organism, particularly a human or animal body, patient or subject, with at least one removal system, preferably a syringe. Preferred according to the invention, the blood is transformed with the at least one nucleic acid molecule within the syringe, the blood cells to be transformed not being previously separated, that is, fractionated, from the other blood components of the removed blood. In an alternative of the method according to the invention, the blood is removed with at least one removal system, preferably a syringe, and subsequently filled into at least one other container. It is preferred according to the invention that the removed blood is transformed in this at least one further container, preferably without previously separating the blood cells to be transformed from the other blood components, that is, as unfractionated blood, preferably as whole blood. In an alternative, blood cells, particularly nuclear cells such as mononuclear cells, PBMC, are separated from other blood components; the blood cells are transformed, and incubated in a medium with or without serum, particularly autologous serum, that were obtained in the abovementioned separation, that is, fractionation, as isolated blood components or in pure, that is, undiluted, serum, preferably pure autologous serum, which was obtained in the abovementioned separation as an isolated blood component.

[0025] It is preferred according to the invention, that at least one transforming nucleic acid molecule is contained in a vector, for example in a plasmid or a virus.

[0026] It is preferred according to the invention that the at least one transforming nucleic acid molecule is functionally connected to at least one regulating element, for example, a promoter, enhancer, or intron, in particular at least one blood cell specific regulating element. In an alternative, the at least one transforming nucleic acid molecule is functionally connected to at least one nucleotide section coding for protein secretion from the cell.

[0027] Preferred according to the invention, the nucleotide molecule to be transformed, particularly DNA or RNA, is if necessary labeled with at least one labeling substance. In an alternative, this labeling substance is used for removing the excess, that is, untransformed, DNA after the conclusion of the transformation.

[0028] Preferred according to the invention, the transforming nucleic acid molecule, particularly DNA or RNA, transforms at least one further material which modulates, preferably increases, the transfection rate and/or the expression rate of the transforming nucleic acid molecule. Preferred according to the invention, the further material fulfills at least one of the following named functions: (a) recognition of the surface of specific cell types, so as to specifically transform these cells; (b) increase of the uptake efficiency into the cell of the transforming nucleic acid; (c) optimizing the nuclear transport of the transforming nucleic acid molecule; (d) increase of the transcription efficiency of the transgene into the cell.

[0029] In a particularly preferred embodiment of the method according to the invention, the at least one nucleic acid molecule, particularly DNA or RNA, is immobilized on at least one solid carrier, in particular large or small beads, for example of glass, on magnetic small spheres, and/or on the wall of the vessel in which the transformation takes place, the syringe in particular being used for transformation.

[0030] The transfection with beads, preferably micro glass beads ("bead transfection") represents a system in which the beads, preferably glass beads, are coated with nucleic acid molecules, preferably with plasmid DNA. In connection with the present invention, "micro glass beads" or "glass beads" are not to be understood solely as the material glass, but also beads of further materials, which are comparable in their function with glass, and in particular can bind covalently and/or non-covalently to nucleic acid molecules, for example, polymeric plastics such as polyethylene, polypropylene, polytetrafluoroethylene, polyacrylates, polyamides, polycarbonates, polyimides, polyacetates, polyolefins, silicones, polysilanes, latex and the like, and/or mixtures thereof. Preferably according to the invention, the beads are also constituted as magnetic beads. The size of the beads or granulate particles is preferably between 2

and 4 mm diameter; however, even smaller particles, in particular larger than 100 µm, can be used, for example, glass powder.

[0031] During the adherence of the cells to these beads, nucleic acids, in particular DNA or RNA, preferably plasmid DNA, are taken up into the cells, get into the cell nucleus and are expressed there. It is provided according to the invention to apply nucleic acid molecules on a solid support, for example on the large or small glass beads and/or on the surface of the removal system, preferably in the form of a syringe, and/or on further vessels which are brought into contact with removed blood or separated blood factors. Nucleated blood cells from the removed blood adhere specifically to these supports, such as in the case of large beads, and/or phagocytose these supports, such as in the case of small beads.

[0032] The coating of the beads with the nucleic acid molecules preferably takes place in a covalent, or else a non-covalent, manner. A covalent, particularly an acid-labile covalent, binding is above all effective if the supports are taken up by phagocytosis. A non-covalent binding is above all effective when the DNA is taken up after adherence.

[0033] Electroporation is provided as an alternative and/or additional transformation method. In a further preferred embodiment of the method according to the invention, the at least one transforming nucleic acid molecule, particularly DNA or RNA, is transformed by electroporation of the blood cells to be transformed. In a preferred embodiment, the transforming nucleic acid molecule contains at least one nucleic acid molecule which induces, represses, and/or regulates the expression of the body's own proteins, for example, in particular consists of at least one antisense construct, RNA element, transposable element, or transcription factor.

[0034] With the application of specific electrical fields, cells are transfected in dependence on the selected electrical parameters for a given quantity. It is provided to transform nucleated blood cells in particular, using electroporation; this occurs in particular without prior purification of the nucleated blood cells from the blood. This means that, particularly advantageously, nucleated cells are transformed in whole blood. It is provided to preferably use "naked" DNA during the electroporation, if necessary

labeled, preferably with biotin, in order to be able to remove excess, i.e., untransformed, DNA again after conclusion of the transformation processes.

[0035] In a particularly advantageous embodiment of the method according to the invention, after successful transformation, the expression of the at least one transgenic gene product, particularly of the therapeutically and/or diagnostically important protein and/or effector in the at least one transgenic blood cell and the secretion from the blood cell into the serum, the at least one blood cell and possibly at least one further untransformed blood cell from the induced blood composition are separated from serum and an induced, preferably cell-free, serum is obtained.

[0036] Particularly preferred according to the invention, the transforming nucleic acid molecule is transformed by means of liposomes, viral vectors, or bound to large and/or small beads, in particular micro glass beads; in particular, if necessary, the at least one transforming nucleic acid molecule is acid-labile bound to the large and/or small beads.

[0037] A subject of the present invention, apart from this, is furthermore also a method of transformation of at least one cell, particularly present in blood, for example blood cells with at least one nucleic acid molecule, the cells and/or the blood cells being brought into contact with the nucleic acid molecules, the cells and/or the blood cells present in the blood are transformed, and stable or transient transformed cells and/or blood cells are obtained. It is here preferred according to the invention for the nucleic acid molecules to be transformed to be bound before the transformation, in particular covalently, in particular in an acid-labile manner, to large and/or small beads, particularly micro glass beads.

[0038] A further subject according to the invention is a method of treatment of the human or animal body, wherein human or animal blood is removed, preferably by means of at least one syringe, and an abovementioned method according to the invention is performed, in order in particular to produce an induced blood composition. According to the invention, the induced blood composition is applied again to the, preferably the same, human or animal body, preferably reapplied. Preferably the induced

blood serum, substantially alone, in particular after separation of the blood cells and/or other blood components such as erythrocytes and/or fibrinogen, is applied in the human or animal body. In an alternative to this method according to the invention, the produced induced blood composition or the induced blood serum is not reapplied in the same human or animal body, but rather is applied in another human or animal body.

[0039] A further subject according to the invention is the use of large and/or small beads such as micro glass beads, upon which nucleic acid molecules are bound, preferably covalently, preferably in an acid-labile manner, for the transformation of blood, preferably of nucleated cells from blood. Preferred according to the invention is the use for the expression and stabilization of proteins and/or effectors in blood, particularly in blood cells. A further subject according to the invention is thus also the use for the transformation of biological cells, particularly eukaryotic cells, particularly preferred plant, fungal or animal cells such as mammalian cells, in particular human cells.

[0040] A further subject according to the invention is finally the use of blood, particularly whole blood, for the transformation of nucleic acid molecules, coding for at least one therapeutically and/or diagnostically important protein and/or effector molecule, in at least one blood cell of the blood, in particular whole blood, for therapeutic, particularly gene therapeutic purposes, preferably for gene therapy and/or the treatment of leukemia, the treatment of traumatic, degenerative, chronically inflammatory diseases of the nervous system, of the motor apparatus or various internal organs. A further subject according to the invention is also the use of nucleotide molecules, in particular coding for at least one therapeutically and/or diagnostically important protein and/or effector molecule, for the transformation of blood, particularly of at least one blood cell contained in the blood, and the expression and possibly secretion of at least one therapeutically and/or diagnostically important protein and/or effector molecule for the abovementioned therapeutic purposes. A further subject according to the invention is also the use of this nucleic acid molecule, in particular coding for therapeutically and/or diagnostically important proteins and/or effector molecules, for the production of a medicament for this transformation.

[0041] Preferred according to the invention, the pharmaceutical production of the at least one, particularly therapeutically and/or diagnostically important, preferably autologous, proteins, takes place by the patient's own blood cells and the subsequent autologous therapeutic application. Alternative thereto, a transformation and incubation of the blood cells, the preparation and application, are provided directly after the removal of the whole blood into a removal system. In particular when the transformation takes place after separation of blood components or respectively the purification of mononuclear peripheral blood cells, PBMCs ("peripheral blood mononuclear cells"), the transformation is preferably performed (a) without growth induction with, for example, mitogenic or growth-stimulating substances such as interleukin-2 (IL-2), or (b) with growth induction with, for example, mitogenic or growth-stimulating substances such as IL-2.

[0042] After growth induction and the transformation preferably with the use of liposomes or by means of electroporation, it is provided that the cells, with or without further growth induction, are further cultured. It is found that in particular the use of electrical fields, in particular in electroporation, stimulates the cell growth and/or cell development. Electrostimulation is therefore also provided as an alternative to the known growth-stimulating substances.

[0043] For the removal of the untransformed nucleic acid molecules, particularly DNA and RNA, after successful transformation, particularly the removal of the DNA from human serum after incubation, according to the invention substantially the following methods are provided: In a preferred alternative, the nucleic acid molecules to be transformed are coupled to a support; after the incubation, the serum containing the desired proteins is removed and filtered, in order to remove the last support and cell residues, the untransformed nucleic acid molecules are removed together with the supports. In a further alternative, labeled nucleic acid molecules are used; after the incubation, additional materials, particularly supports, are added which have affinity for the labeled nucleic acid molecules. In the case of the nucleic acid molecules preferably labeled, according to the invention, with biotin, supports coated with streptavidin are used, which bind the biotin-labeled molecules. The supports are removed from the

serum by centrifugation, or, in the case of magnetic supports, by means of magnetism, the untransformed nucleic acid molecules bound to the supports being purified with at least one filter which has an affinity for the nucleic acid molecules. For example, biotin-labeled nucleic acid molecules bind to a streptavidin-coated filter. With particular advantage, in this alternative both cell residues and also excess, untransformed nucleic acid molecules are removed from the serum in a single step.

[0044] Example 1: Transformation of a blood cell culture by means of DNA coated micro glass beads (bead transfection).

(a) Blood cell culture

[0045] The blood cell culture is a system which is chiefly used in research and development. Blood cell culture acts to culture whole blood. This method is used for "bead transfection" and for toxicity testing of DNA.

[0046] For this purpose, a 48-well culture plate (Nunc, Wiesabaden) is prepared corresponding to the research protocol, in that micro glass beads are used for "bead transfection" or respectively DNA for a toxicity test. Subsequently, non-coagulated whole blood is placed in the cavities; with a 48-well plate, about 1 ml per cavity is used. A portion of the whole blood is centrifuged as a null sample for about 5 minutes at 5,000 × g; the supernatant is then removed and placed in a cavity of a 96-well plate. The plate with the null sample is closed and stored frozen at -80°C. The 48-well plate is likewise closed and is incubated for about 24 hours at about 37°C under 5% CO2 saturation and saturated air humidity. After the incubation, the serum supernatant of the samples in the 48-well cell culture plate is removed and likewise placed in the 96-well plate. The 96-well plate with the samples is stored frozen at -80°C until analyzed.

[0047] In a toxicity test, or respectively pyrogenicity test, the interleukin-1ß content is determined; in the transfection experiments, the content of an expressed reporter protein or target protein is determined.

[0048] For trials in other formats, the volume is respectively correspondingly adapted.

*(b) Production of the plasmid-DNA **pcDNA-IL-1Ra***

[0049] The plasmid *pdDNA-IL-1Ra* contains at least one sequence which codes for a protein ("coding region"), and also a control unit ("promoter"), which is effective in eukaryotic cells. It consists of the coding region, the IL-1Ra-cDNA, in a pc-DNA-1 vector (Invitrogen Company), Karlsruhe). The isolation of plasmid DNA from bacterial suspensions takes place with a commercial system (Quiagen Maxiprep™ endo-free kit™, Quiagen Company) according to the producer's protocol. Quality control of the isolated plasmid takes place in known manner by specific PCR, restriction digestion, and toxicity test or pyrogenicity test respectively in a blood culture.

*(c) Pyrogenicity test of the plasmid **pcDNA-IL-1Ra***

[0050] It is required that the DNA used is not pyrogenic. In a blood cell culture (see (a)) with pyrogenic DNA, the IL-1ß content is on average 2,500 pg/ml. DNA counts as non-pyrogenic or as endotoxin-free when the IL-1ß content does not exceed the lowest standard of the IL-1ß ELISA test, namely 15.2 pg/ml. The results of the pyrogenicity test are shown in Figure 1.

[0051] No increase in the IL-1ß content on incubation with *pcDNA-IL-1Ra* can be detected. The IL-1ß concentrations of the serum supernatant of the dilution series incubated in the blood cell culture are smaller than 15.2 pg/ml, and the DNA does not have pyrogenic action.

(d) Coating of the glass beads

[0052] Plasmid DNA is bound under specific pH and salt conditions to the surface of glass beads. Not all kinds and qualities of glass can bind non-covalent DNA. In the following manner, it is determined whether the support is suitable for non-covalently binding DNA.

[0053] Firstly it is determined how many washing steps are required to remove all the residues such as glass dust and dirt from the beads to be coated. Glass beads of the Roth Company (Karlsruhe) are usually used as the beads, with the following properties:

Size 2.85-3.3 mm

Chemical composition:

SiO_2	68%
CaO	3%
BaO	6%
K_2O	8%
Na_2O	10%
Al_2O_3	1%
B_2O_3	2%

lead-free

[0054] For this purpose, the beads are washed many times in succession, being filled into a 50 ml tube which is filled with phosphate buffer with pH 5.5 (1/15 mol KH_2PO_3, 1/15 mol $NaHPO_3$). The beads are washed by multiple inverting, and the liquid is then decanted.

[0055] It is advantageous to first pre-wash at an extremely low pH. i.e., between 0.01 and 2, or at an extremely high pH, i.e., between 10 and 14, or respectively first at high pH and then at low pH, or first at low pH and then at high pH, in order to attain an increased degree of cleaning of the glass supports. The wash times per wash passage are between 2 min and 1 hour, preferably 30 min. The treatment times with acid and alkali are between 2 min and 1 hour, preferably 30 min. This acid or alkali treatment simultaneously leads to a modification of the glass surface. This pretreatment or modification occurs by the use of conventional organic and/or inorganic acids such as hydrochloric acid, sulfuric acid, nitric acid, phosphoric acid, citric acid, or acetic acid, etc. and also preferably chrom-sulfuric acid, preferably 50% chrom-sulfuric acid (e.g. Merck, Darmstadt, item no. 1.02499.2500, chrom-sulfuric acid, is diluted with Biochrom purest water Ultra Pure Water No. L 0040 to the desired dilution), or alkalis such as sodium hydroxide, potassium hydroxide, ammonia, etc,

[0056] The degree of dirt contamination is determined by photometric measurement of the optical density (OD) at one or more wavelengths between 200 and 800, preferably at 280 nm (OD_{280}).

[0057] The OD_{280} usually remains constant after 4 wash steps (see Table 1), which shows that removable dirt particles are removed from the glass beads.

[0058] The beads are optionally washed in addition with pure water, conductivity up to at most about 1 µS, until the conductivity corresponds to the original conductivity again. The washing step is then repeated once more. This step removes possible residues of ions which can disadvantageously affect the binding of the DNA and a clinical use.

[0059] As binding buffer, ideally a physiological solution such as PBS [phosphate buffered saline] is used, since then for a cell-physiological or clinical use the ionic strength and composition of the buffer do not have to be adapted. A pH gradient of PBS in the pH value region of 5-7 is adjusted and the binding of DNA is tested. For this, the beads are washed 1-10 times, preferably 4 times, with the respective buffer and are incubated with *pcDNA-IL-1Ra* (10 µg/ml PBS) for 90 min. Before and after the individual steps, the purity of the beads and the binding of DNA are monitored by means of OD measurement at 260 and 280 nm.

[0060] Table 1 shows the typical results of an OD measurement after 4 wash steps for removing dirt particles from the glass beads.

Table 1

pH	OD280			
	Washing process			
	1	2	3	4
5	1.3	0.7	0.3	0
5	1.1	0.7	0.2	0
5	1.0	0.8	0.2	0
5.5	0.8	0.6	0.3	0
5.5	0.9	0.7	0.3	0
5.5	1.1	0.6	0.3	0
6	1.0	0.6	0.4	0
6	1.1	0.6	0.4	0
6	0.9	0.6	0.4	0

[0061] For the investigation of the DNA coating of the beads, the beads were washed 4 times with PBS (pH value as given in the table), in order to saturate the beads with the ions from the buffer, and then incubated with DNA containing buffer for different time periods (30, 60, 90, 120, 150 and 180 min). Before and after the individual steps, it was monitored by OD measurement at 260 and 280 nm, firstly whether all the dust particles were removed, and then, how much DNA remained adhered. The pH value of the buffer at which the largest amount of DNA remained adhered was 5.5 (see Figure 2). The incubation time at which the greatest amount of DNA remained adhered was 1.5 hours (see Figure 3).

[0062] Table 2 shows results of OD measurement for inspecting the washing steps and determining the DNA contents in samples from the bead coating.

Table 2

	OD260	OD280	OD260/ OD280	DNA content [µg/ml]
Wash water 1	0.016	0.012	1.26	0.8
Wash water 2	0.011	0.007	1.66	0.6
Wash water 3	0.011	0.006	1.86	0.5
Wash water 4	0.006	0.005	1.22	0.0
pcDNA before incubation	0.112	0.078	1.43	15.6
pcDNA after incubation	0.086	0.047	1.84	14.3
Wash water 5	0.0	0.0	0.0	0.0

[0063] The glass beads for the further studies were washed in 50 ml sterile tubes filled up to 15 ml (about 400 beads) and washed 4 times in PBS with a pH value of 5.5; during the individual wash steps, careful mixing took place. The wash water was monitored by OD measurement at 280 nm for dirt particles. Then plasmid DNA was added to phosphate buffer with a pH value of 5.5 (1.15 mol KH_2PO_3, 1/15 mol Na_2HPO_3), and 10 ml of this solution was added to the washed beads. After 1.5 hours incubation, the DNA solution was removed and the remaining amount of DNA was determined by OD measurement. The beads were again washed with PBS (pH value 5.5), in order to remove unbound DNA.

(e) Sterilization of the beads

[0064] The sterilization is not to damage the DNA and the binding. Accordingly, no steam sterilization (leads to denaturing the DNA) or radiation (induces mutations in the DNA) is used.

[0065] The sterilization of the DNA-coated beads takes place by addition of 40 ml. of 70% ethanol under sterile conditions. After 24 h incubation, the ethanol is removed and the beads are washed 4 times in PBS (PAA Laboratories Co., Cölbe). Ethanol and wash water are monitored by photometric absorption at 260 and 280 nm.

(f) Detection of the DNA on the beads by means of PCR

[0066] Glass beads were coated and sterilized as described above. In order to provide a further unambiguous detection of the binding of the DNA to the glass beads, a sensitive PCR method was developed. For this, a primer was developed by means of the DNASTAR™ software, and the reaction conditions of the primer (annealing temperature, $MgCl_2$ concentration, elongation time, etc.) were optimized.

[0067] In order to dissolve the DNA from the coated beads, a prepared and an unprepared bead were heated in 200 µl TE at 95°C and incubated for 10 min on ice. Thereafter by a comparison with a dilution series of a known concentration of plasmid DNA it was investigated whether DNA can be detected on the beads (see Figure 4). The staining of the bands took place with ethidium bromide. By comparison with the band strengths of the PCR on the bead supernatant with the band strength of known plasmid concentration, the content of *pcDNA-IL-1Ra* in the bead supernatant was determined.

[0068] No DNA could be detected with the non-prepared beads (not shown); DNA is present on the coated beads. After detection with PCR and gel electrophoresis (see Figure 4), it could be calculated that the DNA concentration in the bead supernatant was at least 1 µg/ml. To each bead, 0.2 µg DNA adhered.

(g) Transfection of the cells

[0069] Transfection took place in a blood cell culture. The glass beads coated with DNA were placed in a 48-well cell culture plate MTP (Nunc Company, Wiesbaden) and incubated with 1 ml heparinized whole blood at 37°C under 5% CO_2. After 24 hours' incubation, the total protein content and the content of reporter protein or target protein in the serum supernatant were analyzed.

(h) Determination of the PCR detection limit for plasmid DNA in human serum after transfection

[0070] For the use of the method in clinical practice, it must be ensured that there is no plasmid DNA in the serum. This was tested using PCR.

[0071] DNA polymerase (Taq polymerase) and dNTPs were purchased from the Quiagen Company (Hilden) in "Supermix™" and used. The primer was purchased from Invitrogen (Karlsruhe). Its concentration was adjusted to 20 µmol/l with distilled water.

[0072] For PCR amplification, the following primer pair SEQ ID NO.1/SEQ ID NO 2 was used:

(SEQ ID NO. 1) GCGCTTGTCCTGCTTTCTGTTCTC

(SEQ ID NO. 2) TGGAGTTCCGCGTTACATA

[0073] PCR amplification was performed in 30 cycles of 94°C denaturation (30 sec), 58°C annealing (30 sec) and 72°C elongation (45 sec.). Testing of the amplification took place by means of agarose gel electrophoresis.

[0074] In order to determine the detection limit of plasmid DNA amounts, human serum was mixed 1:1 with a dilution series of plasmid DNA. The minimum plasmid concentration detected by PCR was 10 ng/ml (see Figure 5). A sensitivity of PCR of 1 µg/ml can be determined (see band 2 in Figure 5) by PCR on a dilution series of known plasmid concentration.

[0075] In order to attain a high sensitivity, a "nested" PCR was performed. For performing the "nested" PCR, a further primer pair was used, SEQ ID NO. 3/SEQ ID NO. 4.

(SEQ ID NO. 3) GTCCTGCTTTCTGTTCTCGCTCAG

(SEQ ID NO. 4) AACTAGAGAACCCACTGCTTAC

[0076] The serum was mixed 1:1 with a dilution series of plasmid DNA, in order to detect the detection limit of the PCR. The detectable end concentration of *pcDNA-IL-1Ra* in human serum was 10 pg/ml (Figure 6).

(i) Detection of pcDNA-IL-1Ra in human serum after use of the coated beads

[0077] Human, non-transfected serum Qa mixed with a dilution series of plasmid DNA pcDNA-IL-1Ra and compared with human serum after transfection in a PCR. Using the dilution series of pcDNA-IL-1Ra, the sensitivity of the PCR can be determined; it is 10 pg/ml. Figure 7 shows the analysis of human serum after transfection by means of PCR. No amounts of DNA of 10 pg/ml or more can be detected in the human serum after transfection. This means that no DNA remains in the serum after transfection.

(k) Expression of the transgene

[0078] The target cells were incubated for 24 h with the previously coated beads and then a IL-1Ra and IL-1ß measurement was undertaken.

As controls are:

1. Serum supernatant at start of trial
2. Serum supernatant after incubation with similarly treated but not coated beads
3. Serum supernatant after incubation without beads.

[0079] After analysis of the samples, an increase of the IL-1Ra content in the samples incubated with coated beads was detected in comparison with the samples incubated with non-coated beads and samples incubated without beads (see Figure 8). According to IL-1ß measurement, no increase could be detected; the measurement values lay between 8.32 and 11.51 pg/ml (not shown). This shows that the IL-1Ra production was not brought about by a pyrogenic reaction and must be the consequence of the transient transformation.

[0080] Example 2: Transformation by means of DNA-coated glass beads in a syringe

(a) Preparation of the syringe

[0081] Sterile granulate of glass was used, for example granulate of the Roth Company (Karlsruhe), Item No. 17557.1, or a granulate of the following types:

(1) Roth

Size: 2.85-3.3 mm

Chemical composition (%):

SiO_2	68
CaO	3
BaO	6
K_2O	8
Na_2O	10
Al_2O_3	1
B_2O_3	2

lead free

(2) SiLi 5506/89-6

Size: 2.3-2.5 mm

Material: Borosilicate glass

Treatment: 1. Grinding method

2. Polishing method

Chem. composition (%):

SiO_2	82

Na_2O	2
Al_2O_3	2
B_2O_3	14

Specific weight (kg/dm^3)	223
Hardness (Moh)	7
Linear expansion coefficient (20-300°C)	325
Hydrolytic class (DIN ISO 719)	1
Acid class (DIN 12116)	1
Alkali class (DIN ISO 695)	2
Transformation temperature (°C)	530

(3) SiLi 5004/99-5

Size	2.5 mm
Material:	Soda lime glass
Treatment:	Press method

Chem. composition (%):

SiO_2	67
Na_2O	16
CaO	7
Al_2O_3	5
B_2O_3	3
MgO	2
PbO	free

Hardness (Moh)	greater than or equal to 6

(4) Worf

Size	2-3.5 mm
Material	soda lime glass
Treatment	polished and thermally stabilized

Chem. composition (%):

SiO_2	65
Na_2O	16
CaO	7
Al_2O_3	5
B_2O_3	3
MgO	2

Lead content: free

Density (g/dm^3)	2.54
Hardness (Moh)	6
Hydrolytic class	3
Deformation temperature (°C)	530 ± 10

(5) Duran

Size:	2-3.5 mm
Material:	Borosilicate glass
Treatment:	1. Grinding method
	2. Polishing method

Chem. composition (%):

SiO_2	81
Na_2O, K_2O	4
Al_2O_3	2

B_2O_3	13	
Density (g/dm^3)		2.23
Linear expansion coefficient (20-300°C)		3.25
Hydrolytic class (DIN ISO 719)		1
Acid class (DIN 12116)		1
Alkali class (DIN ISO 695)	2	
Transformation temperature (°C)	525	

[0082] The surface of the granulate is normally modified in a batch process using a commercial chrom-sulfuric acid preparation, as given in Example 1; however, any other acid or alkali mentioned in Example 1 may also be used. The granulate is then rinsed with water in order to wash away the acid/alkali residues. The granulate is then incubated at 121°C under a pressure of 2 bar for at least 20 min, in order to thus sterilize the granulate and saturate it with water. The granulate is then dried at 80°C for 20 min.

[0083] Firstly, washing steps are performed in sterile 50-ml tubes. Then about 150 glass beads are transferred to a plastic perfusor syringe, for example, Type No. 00137 (Becton, Dickinson and Co., Heidelberg) and coated with DNA in the syringe. The DNA solution is drained off and the beads are washed. All further steps are performed under sterile conditions. For sterilization, 70% ethanol is added, the syringe is closed and incubated for about 24 hours. The wash steps with PBS (PAA Laboratories, Cölbe) then follow, PBS being filled above and allowed to run out again. Adapted to the size of the syringe, about 1, 2, 4 or 10 cm^3, or possibly more or less, of the prepared beads are filled into a second syringe. If necessary, the syringe is filled with a sufficient amount of an anticoagulant such as heparin (Liquemin™, Heparin-Natrium™ 2500 I.E.) or Na citrate, as well as the sterilized and modified granulate. This syringe is closed and wrapped in transparent foil.

(b) General use of the syringe

[0084] The user takes the sterile set out of the packaging and removes blood from the patient. The syringe has an opening via a septum in its closure attachment, for puncture by the removal accessory, that is, the needle of the adapter. After removal of the adapter, the septum automatically closes again. After blood removal, the syringe plunger is broken off at a predetermined fracture point. Fig. 12 shows a preferred embodiment of this syringe.

[0085] The syringe with blood is then incubated for 24 hours at about 37-41°C. The incubation takes place lying horizontally. The blood can preferably be transferred and centrifuged. After centrifugation, the plasma is then removed through a sterile auxiliary filter, for example, 0.2 µm.

(c) Experimental use of the syringe for clinical usability

[0086] Blood is removed from a voluntary donor with the prepared syringe. As a control, an ORTHOKIN® syringe (ORTHOGEN Company, Düsseldorf), a perfusion syringe without beads and a 10 ml serum tube (Sarstredt Company, Nümbrecht) were taken as null sample. The null sample was centrifuged at 4,000 × g at 4°C for 10 min, and the serum supernatant was frozen at -20°C and stored. The syringes were incubated at 37°C and 5% CO_2 for 24 hours. After incubation, the serum was removed and centrifuged at 4,000 × g, 4°C, 10 min, in order to remove all blood cells. The supernatant is drawn off with a 20 ml syringe (Sarstedt Company, Nümbrecht), sterile filtered with a 0.2 µm filter (Minisart™, Sartorius Company) and filled into 1.8 ml tubes. The serum is frozen at -20°C and stored for analysis.

[0087] In order to be able to estimate the clinical usability of the system, the contained serum is carefully analyzed. Besides the quality control of the blood product usual in transfusion medicine, such as screening for HCV, HBV, HIV and syphilis, a sterility test and a measurement of transgenic activity were performed. It must be ensured that a usual quality control can be performed and is not disadvantageously affected.

[0088] The use of a syringe as a removal system permits a clinical use. The quality controls of the blood product usual in transfusion medicine, such as screening for HCV,

HBV, HIV and syphilis, give a like result in all tested systems. The sterility test showed that no microorganisms were present in all systems after working up the serum.

[0089] An increase of the IL-1Ra content in the trial according to the invention, in comparison with the null sample and to the ORTHOKIN™ syringe (comparison example) was determined (see Figure 9). On the contrary, no increase of the IL-1ß content can be detected; the measurement values are between 7.2 and 10.9 pg/ml (not shown). This shows that the IL-1Ra production is not brought about by a pyrogenic reaction and must be the consequence of the transient transformation.

(d) Clinical result

[0090] On a group of patients, 18 therapeutic applications were performed with the systems described here, and were clinically evaluated. The patients concerned had cartilage defects in the knee or the ankle joint, due to degenerative and inflammatory diseases of the motor apparatus.

[0091] The assessment of the effectiveness and of side effects was documented radiologically and assessed from WOMAC score, VAS score, and anamnestic data.

[0092] Regarding effectiveness, there can be observed in all the researched and treated patients, in the treated joints in the observation period of a half year, a striking, surprising and highly significant suppression of pain, improvement in mobility, and improvement in general findings. No serious side effects occurred.

[0093] Example 3: Transformation of mononuclear blood cells (PBMC)

(a) Isolation and culturing of PBMCs

[0094] For isolating PBMCs (mononuclear blood cells), a density gradient solution was used. For this purpose, uncoagulated whole blood was diluted to equal parts with culture medium or physiological saline (e.g., Dulbecco's PBS). 50 ml centrifuge tubes were provided with 12.5 ml separation solution (density 1.077 g/ml) and the diluted whole blood was carefully layered over the separation solution (up to about 25 ml per tube). The tubes were centrifuged at 400 × g for 30 min at 15-25°C. After centrifugation, the mononuclear cells form a white boundary layer between the

plasma and the gradient solution. Erythrocytes and granulocytes are in the sediment, and thrombocytes are present in the serum.

[0095] First plasma and then mononuclear cells are carefully removed with a pipette, and are separately transferred to sterile tubes. The cell suspension with mononuclear cells (PBMCs) is mixed with an equal part of culture medium or a physiological saline (Dulbecco's PBS) and was centrifuged off unbraked at 250 × g for 10 min at 25°C. The pellet was resuspended and the cells were washed 2-3 times with cell culture medium RPMI 1640 (with L-glutamine) (Invitrogen, Karlsruhe; Item No. 21875034).

(b) Electroporation

[0096] In electroporation, a cell suspension is subjected to one or more electric pulses in the presence of a DNA solution. Pores are thus produced in the cell membrane, through which the DNA can get into the cell. The formation of the pores is dependent on various factors, and the pores have to be closed again after the electroporation. Temperature and the electroporation medium are particularly decisive for this. After the uptake of the DNA, the DNA migrates into the nucleus and is transcribed in the nucleus. The migration into the nucleus is electrophoretically supported by the pulses. Different pulses are usually applied. The first pulse, or first pulses, are short and strong, that is, they have a high field strength; the following pulses are if necessary weaker and can have different parameters (see below).

[0097] For electroporation of PBMCs, the following conditions are set:

Cell number:	10^4-10^{10} cells per ml, preferably 10^7 cells/ml
DNA amount:	1-100 µg per trial, preferably 20 µg per trial
Field strength:	10-2,500 V/cm, i.e., 4-1,000 V
	with a 4 mm cuvette: preferably 1,500 V/cm,
	i.e., 600 V
Pulse number:	1-100, preferably 10

Pulse duration: 1-1,500 µsec, preferably 0.1 µsec

Pulse spacing: 0.01-1 sec, preferably 1 sec

Capacity: 10-4,000 µF, preferably 1,050 µF

Electroporation medium:

RPMI,

RPMI + 10-30% FCS,

RPMI + 10-30% AP, PBS.

preferably RPMI + 10% AP

(c) Effect of growth-stimulating factors

[0098] The PBMCs are incubated in medium with added growth-stimulating factors. Culturing takes place in RPMI 1640 at 37°C, 5% CO2, and 10% autologous plasma, appropriately with the addition of growth-stimulating substances such as 10-30U/ml of IL-2 (Roche, Mannheim) or 2-10 µg/ml PHA-M (Roche, Mannheim).

[0099] After 3 days of incubation, electroporation is performed. The composition of the medium is: RPMI + 10% AP + 10 U/ml IL-2. The electroporation takes place under the following conditions: 10^7 cells/ml, 20 µg DNA, 1,500 V/cm in 4 mm cuvette, 10 pulses, 0.1 sec pulse duration, 1 sec pulse spacing, and subsequent incubation on ice for 15 min.

[00100] Fig. 10 shows the specific activity of the ß-galactosidase after electroporation of PBMCs with *pVaxLacZ*. There is a clear increase in the specific activity of the ß-galactosidase in samples electroporated with DNA, in comparison with non-electroporated controls, or controls electroporated without DNA.

[00101] Example 4: Production of a glass syringe with granulate

[00102] For the production of the syringe, the surface of the inner structure of the factory-fresh syringe from its original packaging and of the factory-fresh and originally packaged granulate are washed and modified according to Example 1. The syringe is

treated for this by 1-10 times, preferably 3 times, complete filling and ejecting of the acid and/or alkali, and is thereby cleaned or modified. After the last filling, the syringe is sealed below and is incubated in the filled state for 5-30 min with the acid and/or alkali. The syringe piston is then removed and the syringe cylinder is washed through with fresh pure water two through ten times, preferably four times, by complete filling and allowing to run out; care has to be taken that the wash water is completely filled and emptied. The syringe plunger is then dipped in acid and/or alkali and basically washed with distilled water.

[00103] Water residues possibly present in the syringe are sucked out by capillary action by dabbing the Luer connection, in order to ensure rapid drying of the syringe. The piston and syringe, separated from one another, including the glass beads possibly contained therein, are wrapped (Melag, Melaseal) in Melag-foil with indicator field (Melag, Melafol 1502). The thus packaged syringes are dried in a drying closet (Melag dry sterilizer) at 80°C for at least 60 min. The dried, packaged syringes are subsequently autoclaved (Wolf autoclave HRM 242 II) at 132°C for 30 minutes at 2 bar and dried a further time at 80°C for at least 60 min.

[00104] The granulate is washed plural times with salt solution/buffer and is incubated at least 2 hours in the plasmid – salt solution. This plasmid contains at least one sequence which codes for a protein and/or effector molecule, and also at least one regulatory element such as a promoter, which is effective in eukaryotic cells, for example *pcDNA-IL-1Ra*. The freedom from pyrogens of the DNA used is decisive for high protein and/or effector production. The described beads, or respectively granulate, are washed after the incubation and sterilized with ethanol or gas in known manner, preferably as shown in Example 1.

[00105] Before use of the glass beads, heparin (e.g., Liquemin™ N 2500, Heparin-Natrium™ 2500 I.E.) or citrate (e.g., ACDA) is introduced into the syringe in order to prevent a coagulation of the later uptake of blood. The onset of coagulation is also advantageously demonstrated in the working-up of IL-1Ra-containing serum.